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This is a request for filing a PROVISIONAL APPLICATION under 37 C.F.R. § 1.53(b)(2).

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Inventor(s)/Applicant(s)				
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A Combined Computational-Experimental Approach Predicts Human microRNA Targets				
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City: Philadelphia		State: Pennsylvania	Zip Code: 19104 - 6283	Country: US
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Respectfully submitted,

Signature: Artemis Hatzigeorgiou Date: 7/15/03
Typed or Printed Name: Artemis Hatzigeorgiou

Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

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A combined computational-experimental approach predicts human microRNA targets

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Abstract

A new paradigm of gene expression regulation has emerged recently with the discovery of microRNAs, an evolutionarily-conserved class of ~22 nucleotide (nt) RNAs. miRNAs control gene expression by base pairing with miRNA-recognition elements (MREs) found in their messenger RNA (mRNA) targets. Despite a large number of reported miRNAs their mRNA targets remain elusive. Here we use a combined bioinformatics and experimental approach to identify important rules governing miRNA-MRE recognition that allow prediction of human and mouse miRNA targets. We predict mRNA targets for human and mouse miRNAs and provide a strategy to identify mRNA targets for all known miRNAs.

miRNAs are derived from endogenous genes that are initially transcribed as longer RNA transcripts {Lee, 1993 #23} {Wightman, 1993 #39} {Reinhart, 2000 #34} {Lagos-Quintana, 2001 #17} {Lau, 2001 #21} {Lee, 2001 #22} {Mourelatos, 2002 #30}. These transcripts are processed in the nucleus into ~75nt precursors (pre-miRNAs) that fold as single stem-loop structures {Lee, 2002 #24}. Pre-miRNAs are exported to the cytoplasm where the nuclease Dicer excises the mature miRNAs {Hutvagner, 2001 #12} {Lee, 2002 #24}. miRNAs are bound to proteins that belong to the Argonaute family and assemble with other proteins, including the Gemin3 and Gemin4 proteins, to form micro-Ribonucleoprotein complexes (miRNPs) {Mourelatos, 2002 #30}. Dicer also processes another class of ~22nt RNAs termed short interfering RNAs (siRNAs) {Elbashir, 2001 #7} {Hamilton, 1999 #9} from double stranded RNAs {Bernstein, 2001 #3}. Like miRNAs, siRNAs are bound to Argonaute proteins {Hammond, 2001 #11} {Martinez, 2002 #28} and assemble with additional proteins to form RNA-Induced Silencing Complexes (RISCs) {Hammond, 2001 #11}. siRNAs base-pair with their target RNAs with near perfect complementarity and direct target RNA endonucleolytic cleavage {Elbashir, 2001 #7}. This is the mechanistic basis of mRNA destruction in RNA interference {Fire, 1998 #8}. miRNAs function by base pairing with miRNA-recognition elements (MREs) found in their mRNA targets. The critical determinant of miRNA function is the degree of complementarity between a miRNA and its RNA target: if the complementarity is extensive (as is the case with plant miRNAs) the RNA target is cleaved (and the miRNA functions essentially as an siRNA) {Hutvagner, 2002 #13} {Rhoades, 2002 #35} {Llave, 2002 #27} {Tang, 2003 #37} {Xie, 2003 #40}; if the complementarity is partial, translation of the target mRNA is repressed {Olsen, 1999 #31} {Seggerson, 2002 #36} {Zeng, 2002 #48} {Doech, 2003 #47}. In plants, the computational identification of miRNA targets was facilitated by the extensive complementarity between plant miRNAs and their mRNA targets {Rhoades, 2002 #35}. Two plant miRNA targets (mir-171 and miR-162) have been verified experimentally {Llave, 2002 #27} {Xie, 2003 #40}. Two mouse miRNAs (miR-127 and miR-136) also show perfect antisense complementarity with the coding region of a retrotransposon-like gene (Rtl1) {Seitz, 2003 #44}. However, most animal miRNAs are thought to recognize

and attenuate the translation of their mRNA targets via partial antisense complementarity {Lee, 1993 #23} {Wightman, 1993 #39} {Moss, 1997 #29} {Reinhart, 2000 #34} {Olsen, 1999 #31} {Zeng, 2002 #48} {Doench, 2003 #47}. Because of this partial complementarity, simple homology-based searches have failed to uncover targets for miRNAs in organisms other than plants ({Rhoades, 2002 #35} and our unpublished data). The few animal miRNA targets that are known, have been identified in genetic screens. In particular, genetic dissection of the heterochronic gene pathway in *C. elegans* identified the *lin-14* and *lin-28* mRNAs as targets for the *lin-4* miRNA {Lee, 1993 #23} {Wightman, 1993 #39} {Moss, 1997 #29}, and the *lin-41* mRNA as a target for the *let-7* miRNA {Reinhart, 2000 #34}. Importantly, these studies demonstrated that individual MRE sequences are necessary and sufficient to confer miRNA-dependent gene expression regulation in MRE-bearing target mRNAs {Moss, 1997 #29} {Reinhart, 2000 #34}. In *Drosophila*, the novel *bantam* miRNA regulates the pro-apoptotic gene *hid* {Brennecke, 2003 #4}, while the human miR-23 regulates the Hes1 transcription factor {Kawasaki, 2003 #14}. Putative MREs for other miRNAs have been proposed {Lai, 2002 #20} {Xu, 2003 #41} {Lin SY, 2003 #25} {Abrahante JE, 2003 #1}, but these are predominantly based on visual inspection of putative mRNA targets for partial complementarity with miRNAs and lack experimental verification. The rules guiding miRNA:MRE interactions are unknown, making prediction of miRNA targets virtually impossible.

To search for human miRNA targets we employed a bioinformatics approach. We limited our searches to the 3'-UTRs of human mRNAs, extracted from the annotated Reference mRNA Sequences (RefSeq) database (comprising a total of _____ base pairs) {Pruitt, 2003 #43}. Repetitive elements, such as Alu transposable elements that are embedded in a random fashion in ~5% of all human mRNAs, were filtered out before running the searches. We used six miRNAs (*let-7b*, miR-141, miR-24, miR-145, miR-23a and *let-7e*) all of which are 100% conserved between humans and mice. Because there were no known examples of human miRNA:MRE interactions, we could not follow the typical computational approach which is to use known examples to formulate and train an algorithm. Instead, we hypothesized that high affinity interactions, based on binding

energies between two RNAs paired imperfectly, might identify miRNA:MRE interactions. We implemented a modified dynamical programming algorithm that was calculating free energies of both canonical (Watson-Crick) and G-U wobbles base pairs{Tinoco, 1973 #50}. To identify putative MREs, we used a window of 35nt that was "sliding" over the 3'-UTR database and calculated the minimum binding energy between the miRNAs and sequences in the 3'-UTR database. Mismatches were allowed and hits were sorted by lowest binding energies. This analysis revealed ____ hits. Because all miRNAs used for the searches are 100% conserved between humans and mice, it is likely that their mRNA targets, and hence their MREs, are also conserved. ____ hits were found to be conserved between human and mice. Visual inspection of the data showed that one of the predicted MREs for *let-7b* was found in the 3'-UTR of both the human and mouse mRNAs that code for the human/mouse homolog of the *C. elegans* LIN-28 protein, a putative RNA-binding protein (see Figure 1). This finding is particularly interesting because the *lin-28* and *let-7* genes function in the same developmental pathway in *C. elegans*{Moss, 1997 #29}.

Because MREs are necessary and sufficient to confer mi/siRNA-dependent translational repression{Moss, 1997 #29} {Reinhart, 2000 #34}, we reasoned that placement of predicted MREs for specific miRNAs in the 3'-UTR of a reporter construct, followed by transfections in cells expressing the miRNAs that recognize the MREs, should lead to a decrease of the reporter protein levels. We cloned the predicted LIN-28 MRE in the 3'-UTR of a Renilla Luciferase (RL) reporter construct. As a positive control, we generated two RL constructs each bearing in the 3'-UTR one of the two reported MREs for *let-7*, derived from the *C. elegans* LIN-41 mRNA, an experimentally verified *let-7* target {Reinhart, 2000 #34}. As a negative control, the sequence of the LIN-28 MRE was scrambled and placed in the 3'-UTR of RL. We cotransfected the RL-MRE bearing constructs along with a plasmid encoding Firefly Luciferase (FL) in two different cells lines: HeLa cells, a human epithelial cell line and MN-1 cells, a mouse motor neuronal cell line. These cell lines normally express all *let-7* paralogs which are 100% conserved between humans and mice ({Lagos-Quintana, 2001 #17}{Dostie, 2003 #6} and our unpublished data). 18 hours after transfection we quantitated the levels of

normalized RL/FL using standard luminometric assays. As shown in Figure 1 we consistently observe a ~5-fold reduction in the protein levels of RL bearing the LIN-28 MRE versus RL bearing the scrambled MRE (negative control), an effect which is stronger than that of the two positive control MREs derived from LIN-41 (LIN-41a and LIN-41b, Figure 1). Similar results were obtained with both cell lines when the luminometric assays were performed 16, 24 or 48 hours after transfections (our unpublished data). These results confirm the validity of the predicted LIN-28 MRE.

We next wished to determine which of the remainder MREs represented true miRNA targets by investigating the rules that guide miRNA:MRE binding. We hypothesize that such binding is guided by miRNA-associated protein(s) that impose restraints on the position and sizes of loops and nucleotide bulges between miRNAs and their cognate MREs. miRNP proteins and in particular the Argonaute family of proteins represent excellent candidates for guiding such miRNA:target mRNA interaction. Indeed, in a human neuronal cell line, a Gemin4-Argonaute-let-7b containing miRNP associates with endogenous LIN-28 mRNA in polysomes (see accompanying *Brevia* manuscript). To determine the rules of miRNA:MRE binding we generated a series of mutant LIN-28 MREs with varying binding properties between them and the human/mouse let-7b miRNA (Figure 1B). The activity of these MREs was tested as described above, in HeLa and MN-1 cells. As shown in Figure 1C, with the exception of LIN-28-M3 MRE, single nucleotide bulges between let-7b and the LIN-28 mutant MREs that map towards the 5'-end of let-7b, abolish repression of RL expression (mutants LIN-28-M1, -M2, -M4, -M5 and -M6). The single nucleotide bulge of LIN28-M3 MRE is symmetrically placed between the beginning of the loop and the beginning of base pairing between the 5'-most let-7b nucleotide with LIN-28-M3. A similarly placed single nucleotide bulge is found between let-7a and LIN-41a (one of the two LIN-41 MREs, present in the 3'-UTR of the *C. elegans lin-41* mRNA; Figure 1). The activities of both of these MREs are similar (compare LIN-41a to LIN-28, M3 in Figure 1C). These results show that near perfect complementarity between the first ~9 nt (from the 5'-end) of a miRNA and its cognate MRE is required for miRNA function; and the 5' most nucleotide of miRNAs may or may not base pair with MREs (see bindings between LIN-41a or LIN-41b MREs with let-7a in

Figure 1B). We refer to this region of miRNA:MRE binding as the proximal region. Careful analysis of published work on si/miRNAs, provides further support for this claim: *a.* In the experimentally verified MREs for *lin-4* and *let-7*, there is perfect base pairing between the MREs and the first seven or eight (starting from the 5'-end of the miRNA) nucleotides of each miRNA with none or only a single symmetrically placed nucleotide bulge; and the 5' most nucleotide of *lin-4* and *let-7* may or may not base pair with MREs {Moss, 1997 #29}{Reinhart, 2000 #34}. *b.* Two loss-of-function mutants of *lin-4* and *let-7* miRNAs, identified in genetic screens, are caused by single-point mutations mapping in the first 6 nucleotides in both miRNAs and are predicted to disrupt base pairing in the proximal region{Lee, 1993 #23}{Moss, 1997 #29}{Reinhart, 2000 #34}. *c.* The 5'-end of siRNAs sets the ruler for target RNA cleavage, implying that recognition of the 5'-end of siRNAs is essential for their function{Elbashir, 2001 #7}. *d.* A genetic, single-point mutation, present in the MRE of the *Arabidopsis PHAVULOTA (PHV)* mRNA, disrupts base pairing with the fifth nucleotide of its cognate miR-165/166 miRNA and reduces dramatically the miR-165/166-mediated cleavage of the mutant *phv* mRNA{Tang, 2003 #37}. *e.* Single point mutations mapping in the first 7 nucleotides of an siRNA abolish siRNA activity, whereas point mutations mapping towards the 3'-end of the siRNA have no or much smaller effects{Amarzguioui, 2003 #2}.

In contrast to the strict requirements for base-pairing at the proximal region, nucleotide bulges between LIN-28 mutant MREs and the 3'-end of *let-7b*, (a region that we refer to as the distal region), are tolerated and decrease by ~2-fold the activities of the mutant LIN-28 MREs (Figure 1; LIN-28-M7, -M8 and -M9). The activity of LIN-28-M10, which bears a single nucleotide mismatch away from the loop and close to the 3'-end of *let-7b* is essentially the same with that of the wild-type LIN-28 MRE. We next determined the requirements for the size and position of the loops between *let-7b* and mutant LIN-28 MREs. The optimal loop length found in the wild-type LIN-28 MRE is 5nt. As shown in Figure 1, LIN-28 mutant MREs with single, symmetrically placed loops varying in size from 2nt to 4nt were still active (LIN-28-M12 to -M14), while a single nucleotide substitution of the LIN-28 loop had the same activity as the wild-type LIN-28 MRE (LIN-28-M11). However, LIN-28 mutant MREs with loops longer than 5

nucleotides were unable to repress the Renilla luciferase activity (LIN-28-M15, -M16). Finally, mutant LIN-28 MREs were designed that allowed for a single let-7b loop of varying sizes. As shown in Figure 1, MREs with a 9nt or 7nt let-7b loop were active (LIN-28-M17, -M18) but MREs with a let-7b loop of less than 5nt were inactive (LIN-28-M19 to -M21). In fact the activity of LIN-28-M18 MRE is identical to the wild-type LIN-28 MRE, and resembles the binding characteristics between the *C. elegans lin-4* miRNA and its *lin-28* mRNA target{Moss, 1997 #29}.

These experiments demonstrate that there are indeed rules that govern miRNA:mRNA interactions, which may be generally applicable. These rules are summarized in a schematic form in Figure 2. We note that the repressing properties of a miRNA may depend on the way it interacts with its mRNA target. A miRNA:MRE interaction with a single nucleotide loop of optimal length is more potent (LIN-28, wt; Figure 1) than two small opposing loops (LIN-41b; Figure 1). This finding may explain the requirement, for optimal repression, of two MREs for *let-7* in the 3'-UTR of the *C. elegans lin-41* mRNA{Reinhart, 2000 #34}. On the other hand, a single MRE for *lin-4* in the 3'-UTR of the *C. elegans lin-28* mRNA suffices because it contains a single, 6nt loop{Moss, 1997 #29}. The degree of miRNA-mediated translational repression may ultimately depend on additional factors such as the miRNA and target mRNA concentrations and the accessibility of MREs. The finding of a let-7b MRE in the 3'-UTR of the human/mouse LIN-28 mRNA and the fact that endogenous human LIN-28 mRNA associates with a let-7b-containing miRNP in polysomes (see accompanying paper) strongly indicate that human let-7b and LIN-28 are part of the same pathway, which may be functionally related to the *C. elegans* heterochronic gene pathway. The *C. elegans lin-28* mRNA is predominantly regulated by *lin-4* {Moss, 1997 #29}. Although a direct role for *let-7* in the regulation of *C. elegans lin-28* mRNA has not been shown, *lin-28* is also regulated by a *lin-4* independent pathway{Seggerson, 2002 #36}. There are four *let-7* paralogs in *C. elegans* and it is possible that one of them regulates *lin-28*.

We applied the miBRs to the putative MREs that our initial algorithm had predicted and identified five (in addition to LIN-28) that followed them. We tested these MREs

(Figure 3A) as well as others that did not abide to the miBRs using the luciferase based assay as described above. The cognate miRNAs (miR-141, miR-24, miR-145, miR-23a and let-7e) for these MREs are present in both HeLa and MN-1 cells{Lagos-Quintana, 2001 #17}{Mourelatos, 2002 #30}{Lagos-Quintana, 2002 #19}{Dostie, 2003 #6} (and our unpublished data). miR-141 was originally cloned from mouse{Lagos-Quintana, 2002 #19}. Human miR-141 containing two additional terminal nucleotides has also been cloned from human colonic mucosa and deposited in the Genbank database as miR-157 (accession AJ535825). We have cloned miR-141 from HeLa and MN-1 cells and confirmed the presence of the two additional nucleotides as shown in Figure 3A (our unpublished data). The predicted MREs that did not abide by the miBRs, were unable to suppress the expression of luciferase (Table1 and our unpublished data). In contrast, all MREs that abided by the miBRs, suppressed the expression of luciferase (Figure 3B). A MRE for miR-141 (miR-157) is present in the 3'-UTR of both human and mouse mRNAs coding for the CLOCK transcription factor, which is critical for circadian rhythms. Two identical MREs for miR-24 are present in the 3'-UTR of both human and mouse mRNAs coding for Mitogen-Activated Protein Kinase 14 (MAPK14, also known as p38 α kinase; Figure 3C). MAPK14 has pleiotropic cellular effects; it is a key regulator of stress-induced signaling, cell proliferation and apoptosis and is required for placental and heart development and erythropoiesis (see OMIM entry 600289). A MRE for miR-145 is found in the 3'-UTR of both human and mouse mRNAs coding for a hypothetical 501 amino-acid protein termed FLJ21308 in humans and D13Ertd275e in mouse. FLJ21308 contains a putative poly (ADP-ribose) polymerase catalytic domain, suggesting that it may function in chromatin modification by ADP ribosylation. For let-7e and miR-23 miRNAs, MREs were found in the 3'-UTR of human mRNAs coding for the structural maintenance of chromosomes 1-like 1 protein (SMC1L1) and a 324 amino-acid hypothetical protein termed FLJ13158, respectively. SMC1L1 functions in sister chromatid cohesion during mitosis (see OMIM entries 606462 and 300040). The FLJ13158 protein contains a 120 amino-acid domain of unknown function (termed the DUF738 domain), which is highly conserved in worm, fly, rodent and human proteins. It is tempting to speculate that the two hypothetical proteins whose expression is potentially regulated by miR-145 and miR-23, function in development. If their expression were

developmentally restricted (as is the case for the *C. elegans* LIN-28, LIN-14 and LIN-41 proteins, whose mRNAs are regulated by miRNAs) it might explain why they have escaped detection.

The FLJ13158 mRNA may be regulated by miR-23, a miRNA that was recently shown to play a role in the differentiation of human NT2 neuronal cells{Kawasaki, 2003 #14}. In the same study miR-23 was reported to recognize a MRE within the coding region of the Hes1 transcription factor mRNA{Kawasaki, 2003 #14}. The miR-23:Hes1 interaction does not abide by the miBRs that we have established in this report; in particular there is no central loop and there are many nucleotide bulges or mismatches at the proximal region{Kawasaki, 2003 #14}. Importantly, placement of a single Hes1 MRE at the 3' UTR of a Renilla Luciferase construct did not affect the activity of luciferase{Kawasaki, 2003 #14}, exactly as our rules would have predicted. However, placement of five copies of Hes1 MREs repressed the activity of luciferase{Kawasaki, 2003 #14}. There are two possible mechanisms that may explain this discrepancy. Our validation assay for putative MREs utilizes a single MRE in the 3'-UTR of the RL reporter, which is under the control of the relatively strong Herpes simplex thymidine kinase promoter. We deliberately chose to insert a single MRE to avoid extraneous effects of longer sequences that may arise when multiple MRE copies are used. At the same time, our assay may not be sensitive enough to detect weaker miRNA:MRE interactions that may become apparent when multiple MREs are used. This may be true for miRNAs that are expressed at low levels or for low-affinity miRNA:MRE interactions. Many miRNAs are surprisingly abundant{Lim, 2003 #49} and we expect that a single MRE should suffice to detect high affinity miRNA:MRE interactions. This appears to be the case with the miR-23:FLJ13158 (Figure 3B), but not with the miR-23:Hes1 interaction{Kawasaki, 2003 #14}. Another possibility is that there may exist additional rules governing miRNA:MRE interactions, especially those occurring within the coding regions of target mRNAs, as is the case for Hes1. Further work is required to address these issues and to determine how miRNAs recognize putative MREs in regions other than the 3'UTRs of mRNAs. Nevertheless, this study provides a strategy to predict RNA targets for all known miRNAs, and describes the first set of human miRNA targets.

We anticipate that these findings will facilitate the functional characterization of miRNAs and the genes that they regulate.

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References**Notes & Acknowledgments**

Sequences, constructs and detailed experimental procedures are available upon request (mourelaz@uphs.upenn.edu). We are grateful to Drs. H. Kazazian and J-C Oberholtzer for critical review of the manuscript. Supported by NIH grants to MK (5T32-AR07442), PN (5T32-AG00255) and ZM (NS02199) and by a University of Pennsylvania Genomics Institute award to AH and ZM.

Figure Legends

Figure 1

Experimental verification of a predicted miRNA recognition element (MRE) and of the miRNA Binding Rules. **A.** Schematic representation of the reporter construct; red: coding region. **B.** Potential base pairing between predicted MREs derived from the indicated mRNAs (black) and their cognate miRNAs (blue); wt: wild-type sequence. Nucleotides forming potential bulges between LIN-28 MRE mutants and let-7b miRNA are shown in red. **C.** HeLa human cells (blue bars) or MN-1 mouse cells (orange bars) were cotransfected with Renilla Luciferase (RL) constructs bearing the indicated MREs in the 3'-UTR, along with Firefly Luciferase (FL). Results shown are average values (with standard deviations) of normalized RL/FL activities obtained from six separate experiments.

Figure 2

Schematic representation of miRNA:MRE bindings. Blue: miRNAs; red: MRE. P: Proximal (relative to 5'-end of miRNA) region of miRNA:MRE binding; D: Distal region of binding; L: loop. **A.** Double opposing loops; loop length = 2 to 3nt. **B.** Single MRE loop; length = 2 to 5nt. **C.** Single miRNA loop; length 6 to 9nt. Proximal binding characteristics: \geq 7nt base pairing between miRNA and MRE; the 5' most nucleotide of the miRNA may or may not base pair with MRE; one symmetric nucleotide bulge allowed. Distal binding characteristics: \geq 5nt base pairing between miRNA and MRE. Nucleotide bulges allowed. In A and B the last (towards the 3'-end) nucleotides of the miRNA may or may not base pair with the MRE.

Figure 3

Predicted miRNA targets. **A.** Potential base pairing between predicted MREs derived from the indicated mRNAs (black) and their cognate miRNAs (blue). Accession numbers

(Human/Mouse): LIN-28 (NM_024674/ NM_145833); CLOCK (AF011568/ NM_007715); MAPK14 (NM_139012/ NM_011951); FLJ21308 (NM_024615/ BC021315); FLJ13158 (NM_024909); SMCL1 (NM_006306). Numbers refer to nucleotide positions based on the human mRNAs. **B.** HeLa (blue bars) or MN-1 (orange bars) cells were cotransfected with Renilla Luciferase (RL) constructs bearing the indicated MREs in the 3'-UTR, along with Firefly Luciferase (FL). Results shown are average values (with standard deviations) of normalized RL/FL activities obtained from six separate experiments. **C.** Schematic representation of MAPK14 MREs bound to miR-23. Red: 5'-UTR and coding region.

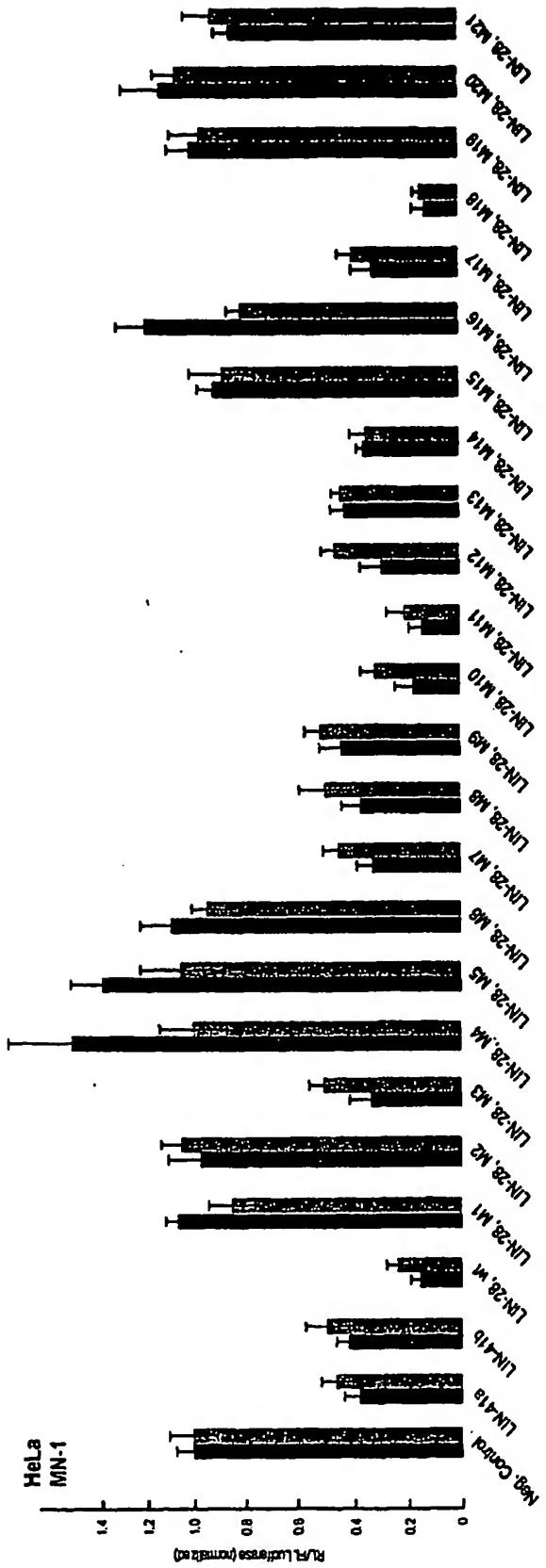
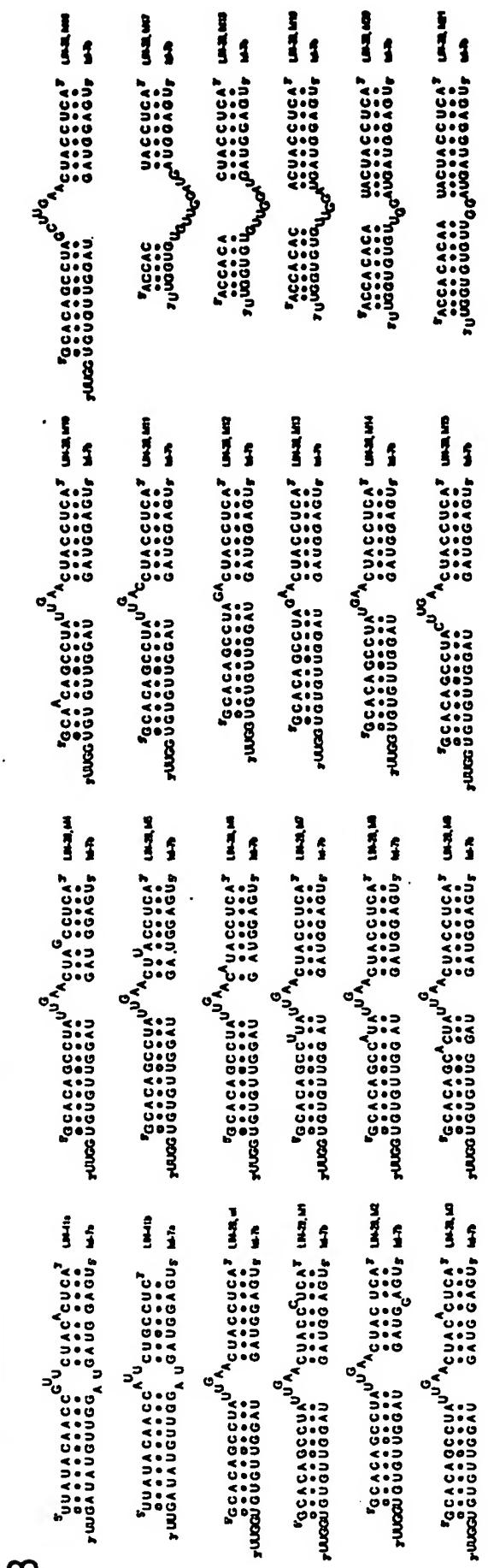


Figure 2

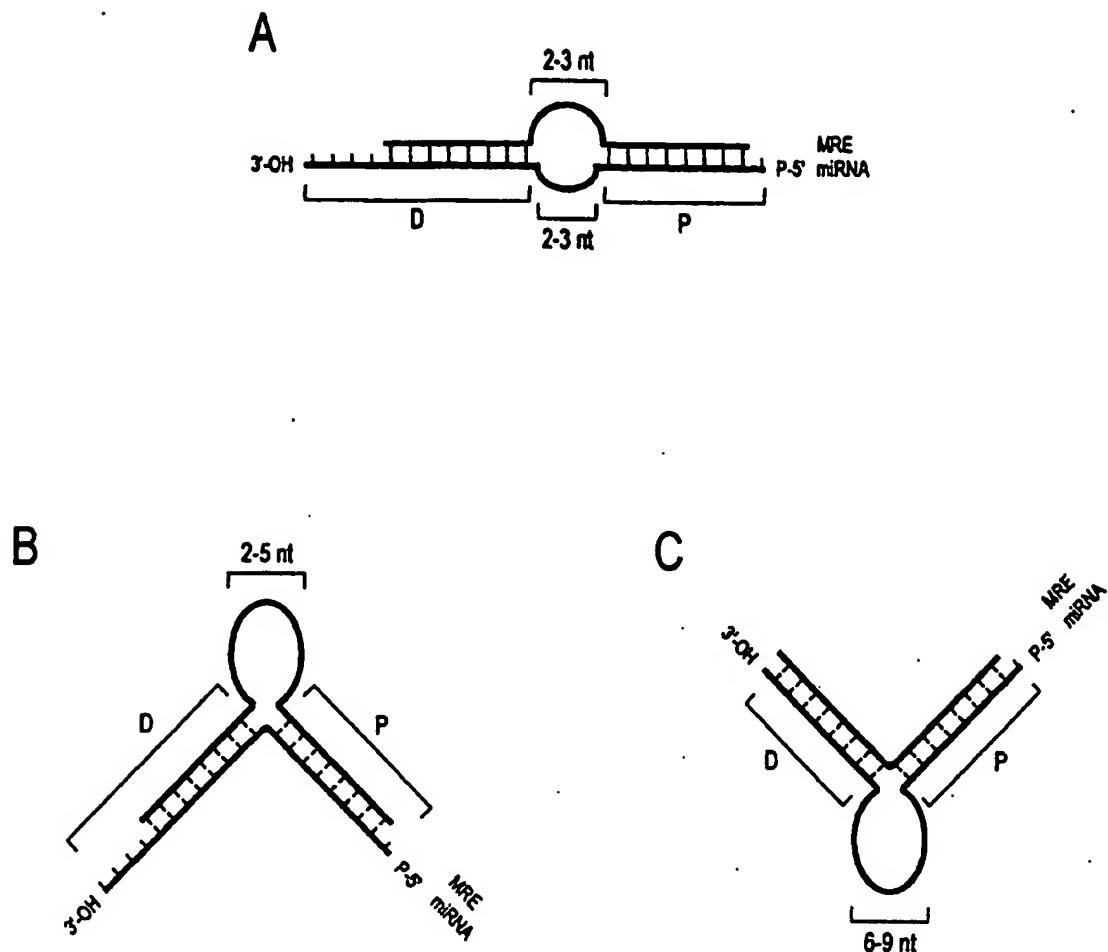
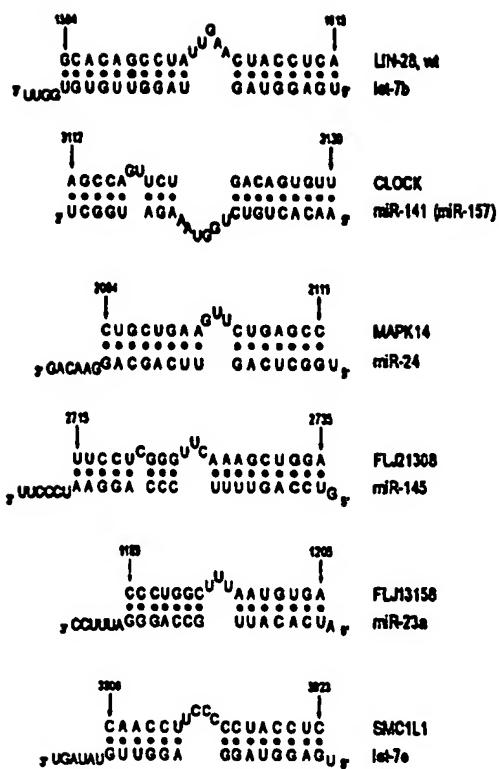
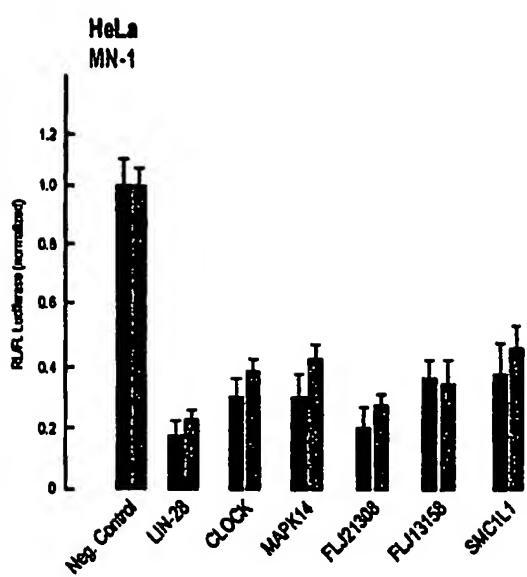


Figure 3

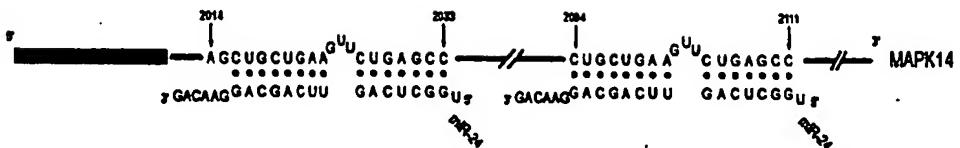
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